Pure Insulin Nanoparticle Agglomerates for Pulmonary Delivery

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Abstract

The advent of nanotechnology has reignited interest in the lungs as a major route of drug delivery for both systemic and local treatments. The large surface area of the lungs and the minimal barriers impeding access to the lung periphery make this organ a suitable portal for a variety of therapeutic interventions. Nanoparticles provide new formulation options for both dispersed liquid droplet dosage forms such as metered dose inhalers and nebulizers, and dry powder formulations. Nanoparticle formulations have many advantages over traditional dosage forms, such as enhanced dissolution properties and the potential for intracellular drug delivery. Specifically, pure drug nanoparticles, polymeric nanoparticles, polyelectrolyte complexes, and drug-loaded liposomes offered some encouraging results for delivering drugs to and through the lungs. Methods are being investigated to produce nanoparticles with properties suitable for improving access to the peripheral lung. Techniques such as spray drying and supercritical fluid extraction have been employed to produce nanoparticle formulations for pulmonary delivery. In Chapter 1, the benefits of nanoparticle formulations and current progress are compared in light of the practical encumbrances of producing formulations, and possible toxicological effects of these materials.

In Chapter 2, a novel, nanoparticle-based insulin formulation is described for the treatment of diabetes. Diabetes is a set of diseases characterized by defects in insulin utilization, either through autoimmune

destruction of insulin-producing cells (Type I) or insulin resistance (Type II). Treatment options can include regular injections of insulin, which can be painful and inconvenient, often leading to low patient compliance. To overcome this problem, novel formulations of insulin are being investigated, such as inhaled aerosols. Sufficient deposition of powder in the distal regions of the lung to maximize systemic absorption requires precise control over particle size and density, with particles between 1 and 5 μm in aerodynamic diameter being within the respirable range. Insulin nanoparticles were produced by titrating insulin dissolved at low pH up to the pI of the native protein, and were then further processed into microparticles using solvent displacement. Particle size, crystallinity, dissolution properties, structural stability, and bulk powder density were characterized. The work described in Chapter 2 demonstrates that pure drug insulin microparticles can be produced from nanosuspensions with minimal processing steps and with suitable properties for deposition in the peripheral lung.

Dedicated to my family,

whose boundless faith and encouragement

have enabled my success.

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1. Nanoparticle Formulations in Pulmonary Drug Delivery

The lungs are perhaps the most historic portal for drug delivery. As early as 1500 B.C.E., the ancient Egyptians inhaled vapors to treat a variety of diseases.[1] Unfortunately, the lungs were soon forgotten as a major route of drug delivery, and it was not until the early 1950s that serious consideration of the lungs was resurrected with the invention of the first metered dose inhaler (MDI). It was used to locally administer albuterol to treat asthma,[1] and offered little precision in control of dosing. Fortunately, this was not a major concern due to albuterol's wide therapeutic window when used as an anti-asthmatic.[2]

Today, researchers have made great strides in the development of precise pulmonary drug delivery technologies, both in terms of inhaler design and advances in particle engineering. Some of the most promising advances have manifested from applying nanotechnology to particle engineering.[3] These have led to several innovative systemic delivery formulations and new treatment paradigms. Here, the benefits of nanoparticle formulations are presented in the context of how they relate to local and systemic pulmonary drug delivery. In addition, specific formulation methods are presented to illustrate the many strategies available to engineer nanoparticles suitable for delivery to the peripheral lung.

1.1. Pulmonary Physiology and Drug Absorption

The primary functions of the lungs are to enable gas exchange between the blood and the external environment, and to maintain homeostatic systemic pH. The respiratory system is composed of the trachea, which bifurcates into the bronchi. The bronchi continue to branch into smaller bronchioles, and ultimately the terminal bronchi, which end with the alveolar sac. The conducting airways are lined with ciliated columnar epithelium, which transition to a cuboidal shape approaching the distal airways. The lumen of the bronchial airways is lined with a thin layer of serous fluid, upon which floats a layer of mucus, which helps to entrap aerosolized particles. The coordinated, rhythmic beating of the cilia constantly moves this mucous layer toward the proximal airways, where it is either swallowed or expectorated (mucociliary clearance). Particles settling in the peripheral lung have been reported to have a residence time of about 24 hours in a healthy adult patient.[1]

The alveolar epithelial surface is primarily composed of type I pneumocytes, which share a basement membrane with the pulmonary capillaries. The alveoli also contain type II pneumocytes, which secrete lung surfactant to prevent alveolar collapse, and macrophages, which are responsible for clearing large particles. There are approximately 300 million alveoli in the lungs, with a combined surface area that is greater than 100 m^2, and with an alveolar epithelium as thin as 0.1 μm.[1,4] This large surface area, combined with an extremely thin barrier between the pulmonary lumen and

the capillaries, creates conditions that are well suited for efficient mass transfer.[4]

Particle deposition in the lungs occurs by inertial impaction, sedimentation, or diffusion. Particles larger than 10 μm in diameter are generally subject to inertial impaction in the oropharyngeal region, or sedimentation in the bronchial region, where delivered drug may be expected to have little systemic therapeutic effect.[4-6] At the other extreme, particles with diameters substantially smaller than 1 μm are less likely to reach the alveolar region, but are not likely to deposit and thus are exhaled. Particles with aerodynamic diameters between 1-5 μm will bypass deposition in the mouth and throat and efficiently deposit in the lung periphery.[1,5-7]

Once deposited, drugs encounter a variety of physicochemical and biological barriers. Inside the peripheral lung, particles must dissolve and drug must diffuse through the epithelial barrier and into the blood stream; however, larger particles that dissolve slowly are still subject to mucociliary clearance and phagocytosis by alveolar macrophages. Penetration enhancers can sometimes be incorporated into pulmonary formulations to enhance systemic bioavailability, but the long-term safety of these compounds still needs to be evaluated.[1] Molecules such as small peptides may be subject to enzymatic degradation in the lungs, although the environment is much more hospitable to proteins and nucleic acids than the gastrointestinal tract. Additionally, as the molecular mass of a peptide

increases, its tertiary and quaternary structures become more complex, which reduces peptidase activity on the protein.[1] In addition, cyclization of peptides can reduce susceptibility to degradation by peptidases.[8] Finally, the transport of proteins tends to decrease as molecular weight increases, with proteins greater than 150 kDa having a difficult time accessing systemic circulation. Despite these encumbrances, pulmonary delivery may well represent the ideal route to deliver peptides and small protein therapeutics.

The lungs are suitable for both local and systemic drug delivery. There are many local diseases of the lung that are prime candidates for inhalation therapy, such as asthma, emphysema, chronic obstructive pulmonary disease (COPD), cystic fibrosis, primary pulmonary hypertension, and cancer.[2] Treating these diseases locally is advantageous since the drug avoids first-pass metabolism and deposits directly at the disease site. This type of application of the drug to the lung epithelium also eliminates potential side effects caused by the high systemic concentrations typical of conventional delivery methods, and can reduce costs because smaller doses can be used.[2] The benefits of systemic drug delivery through the lungs include a rapid onset of action and an increased bioavailability over oral formulations, especially for peptide drugs.[9,10] Several companies have capitalized on these perceived benefits to develop inhalable forms of insulin that have the potential to rapidly control postprandial hyperglycemia.[11-13] Despite the lack of

commercial success of inhaled insulin thus far, much excitement surrounds the potential medicinal benefit of inhaled therapeutics.

1.2. Types of Inhalers

There are currently three major types of inhalers used for pulmonary drug delivery; nebulizers, metered dose inhalers (MDIs), and dry powder inhalers (DPIs). All three types of devices use different delivery mechanisms, and hence require different types of drug formulations.

Nebulizers represent an historic technology for delivering drugs to the lungs. Air-jet nebulizers use compressed jets of high velocity air to shear a bulk suspension or solution into a liquid film at the spray nozzle.[14] The film then collapses under surface tension,[14] forming droplets that are aerosolized and inhaled by the patient. Ultrasonic nebulizers utilize a vibrating piezoelectric crystal, which causes cavitation bubble formation at the surface of the solution or suspension.[14] This generates a dense mist when droplets effervesce from the turbulent medium,[14] which is inhaled by the patient. Nebulizers produce ultra fine droplets (~1 μm) with a high degree of polydispersity, and particles larger than the droplet size have been reported not to be aerosolized.[14] For this reason, nebulizers may not be appropriate for delivering large particles to the lungs.

Metered dose inhalers are pressurized vessels that contain drug that is either dissolved or suspended in a liquid propellant (typically

hydrofluoroalkanes, or HFA).[15] When actuated, the device releases a metered volume of drug and propellant through a valve system.[16] Although dosing with MDIs is typically more reproducible than DPIs, metered dose inhalers are generally more difficult to use because they require coordination between actuation and inhalation in order to ensure optimal deposition of drug in the lungs.[15,17] For this reason, patients and physicians sometimes need to undergo training in order to develop the proper technique.[17] Another disadvantage of MDIs is their use of HFA propellants, which have been linked to global warming.[15] MDIs also typically contain surface-active agents like surfactants, which may impact lung performance.[15]

Dry powder inhalers are breath-actuated devices that deliver a dry powder drug through shear-induced aerosolization.[16] For this reason, the actual dose delivered from a DPI is highly dependent on the inspiratory flow rate, and can sometimes be difficult to replicate.[15,17,18] Additionally, particles within a DPI tend to agglomerate due to electrostatic interactions and/or hygroscopic phenomena, thereby inhibiting aerosolization. Despite these disadvantages, DPIs are generally easier to use than other types of inhalers since they do not require coordination of actuation and inhalation, and they do not use liquid propellants.[15,19] There are three typical DPI device categories: the single-unit dose inhaler, the multi-dose reservoir inhalers, and multi-unit dose inhalers.[20] Single-unit dose inhalers require the patient to load a gelatin capsule into the device before each use, which is broken upon actuation.

Multi-dose reservoir inhalers contain a bulk supply of drug in which individual doses are released with each actuation.[20] This gating mechanism of action helps to minimize the flow-dependent dosing that occurs with other DPIs. Another variation of the reservoir inhaler is the multi-unit dose inhaler, which contains multiple sealed blisters that are individually broken upon actuation.[20]

In addition to the three major inhaler types, inhalers that utilize eletrohydrodynamic spray, or electrospray, are also being investigated. In this mechanism, nebulization of the liquid droplets relies solely on electric charging and dispersion occurs due to the Coulombic forces between droplets.[21] This technique has been shown to retain the structure of proteins, and has been considered as a method to deliver insulin to the lungs for the treatment of diabetes.[21] Additionally, electrospray offers good control over droplet size distribution, being able to approach monodispersity.[22,23] One drawback to electrospray is that highly conductive solutions, such as salt solutions, may be too conductive (and thus will not hold a charge) to reach the target droplet size.[22]

1.3. Advantages of Nanoparticle Formulations in Pulmonary Drug Delivery

In a 1959 lecture, Richard Feynman proposed that, in the future, small machines will be used to make smaller machines, and these in turn will be used to make even smaller machines, all the way down to the atomic level.[24]

Today, Professor Feynman's vision has been partially realized with the advent of nanotechnology. Advances in nanoscience have propelled innovations in a number of scientific disciplines, including medicine and pharmaceutical formulation. Nanotechnology has the potential to revolutionize medicine, and has already presented new regulatory challenges.[25,26] Innovations are occurring rapidly, as demonstrated by the exponential increase in nanotechnology-related pharmaceutical patents over the past 15 years.[27]

Drug nanoparticle formulations are usually created in one of two ways. Particles may be precipitated out of solution (bottom-up), or they are milled from larger particles (top-down).[28] In both mechanisms, the total surface area increases, which increases the free energy of the particles. The system compensates for this increase in free energy by dissolving crystalline nuclei and precipitating onto other particles in a process known as Ostwald Ripening,[29] or by agglomerating smaller particles. Generating stable nanoparticle colloids typically necessitates the use of surfactants, which decrease the surface tension at the particle surface and thereby help to reduce the increase in free energy.[28]

Nanoparticle pharmaceuticals offer several advantages over formulations containing larger particles. For example, as the size of a particle decreases, a greater number of its molecules will be found at its surface rather than inside the particle,[3] giving nanoparticles a large surface area to

volume ratio.[6] This increase in total surface area leads to an increase in dissolution velocity, as described by the Noyes-Whitney equation.[30] Additionally, the saturation solubility of a particle increases as the particle size decreases, which is described by the Kelvin and Ostwald-Freundlich equation.[30] Interestingly, this size-dependence only becomes apparent after the particle size falls below approximately 1 μm,[30] making it entirely unique to nanoparticles. These phenomena make nanoparticle formulations a highly effective means to enhance mass transfer from the particle into the surrounding medium.[30] For this reason, nanoparticle formulations have been used to enhance the bioavailability of insoluble hydrophobic drugs.[3] By suspending the drugs as nanoparticles, one can achieve a dose that is higher than that of a solution, which is thermodynamically limited by the aqueous solubility of the drug.[6]

These properties of nanoparticles can be exploited in many ways to enhance a drug formulation. For example, it is sometimes desirable to increase the octanol-water partition coefficient (log P) of drugs by modifying their structures with the addition of an aliphatic tail group. Bhandari, *et al.* demonstrated this through ketorolac esterification with a fatty acid to enhance permeation though the skin, which is a hydrophobic barrier.[31] Similar prodrug modifications have been used in other studies to the same effect.[32-35] Several studies have demonstrated that fluorinated nicotinic acid esters have an increased solubility in perfluorooctyl bromide, which can be used as a delivery

vehicle for drugs to the lungs.[36,37] Lehmler, *et al.* demonstrated that this prodrug strategy in conjunction with the perfluorocarbon delivery vehicle increased the partition of the lipophilic drug into lung epithelial cells.[37] A nanoparticle formulation technique could be used to counteract the decrease in aqueous solubility that comes with the addition of hydrophobic groups to increase the local bioavailability of the drug.

In addition to the enhanced mass transfer properties offered by nanoparticles, several studies have demonstrated that nanoparticles bind to and can be internalized by a variety of cell types.[38-42] Davda, *et al.* showed that vascular endothelial cells rapidly internalize nanoparticles into the cytoplasm.[39] Another study demonstrated that pulmonary epithelial cells internalize particles 0.5 μm or smaller 10 times more than 1 μm particles and 100 times more than 2 or 3 μm particles.[40] These studies suggest that nanoparticle formulations may be an effective way to enhance drug internalization by cells.

1.4. Nanoparticle Processing Methods for Pulmonary Drug Formulations

Many chemical processing technologies have been used to produce drug nanoparticles suitable for pulmonary delivery. Some processes that are currently under investigation involve wet milling,[28,43] supercritical fluid extraction,[44] spray drying,[45,46] electrospray,[21-23] high-pressure homogenization,[47] and recrystallization via solvent displacement.[48] Wet

milling is a process that utilizes either ceramic or metallic milling media to grind a suspension of insoluble drug and surfactant. Merisko-Liversidge, *et al.* have shown that this technique can be used to produce Zn insulin nanoparticles using a roller mill that contains ceramic milling media and surfactants.[43] The authors reported producing insulin nanoparticles less than 200 nm in size.[43] Wet milling has also been used to formulate budesonide for nebulized delivery to the lungs.[28] Use of this technique for other poorly-water soluble drugs has been vindicated by SkyePharma, as evidenced by a recent patent.[49] Spray drying is a process that forces fluid through a nozzle, producing a mist that is dried to produce a fine powder. The technique employs a variety of different types of nozzles, some of which use ultrasound or air-jet shear to nebulize drug suspensions. Supecritical fluid extraction is a technique that is currently being developed for use in nanoparticle drug formulations. It uses supercritical fluid to extract a solvent from a drug emulsion or solution, leaving behind a suspension of drug particles.[44] These processes are advantageous because they generally offer better scalability, and are therefore industrially relevant. Unfortunately, some processes (such as spray drying) often utilize cosolvents to improve drying and/or large amounts of excipient to stabilize the drug and to maintain powder properties.

In addition to chemical processing technologies, multiple recent studies have examined different polymeric nanoparticle fabrication methods as applied to pulmonary drug formulations.[50-55] These techniques generally

involve polyelectrolyte complex formation, double emulsion/solvent evaporation techniques, or emulsion polymerization techniques. Polyelectrolyte complexes use oppositely charged polymers to entrap drugs into a polymeric matrix nanoparticle, which then releases the drug either through polymer degradation or drug diffusion. Double emulsion/solvent evaporation techniques involve dissolving the drug and polymer in an organic solvent, which is then emulsified in an aqueous solution. The organic solvent diffuses out of the polymer phase and into the aqueous phase, and is then evaporated, leaving behind drug-loaded polymeric nanoparticles. Emulsion polymerization is similar to emulsion/solvent evaporation except that monomer is emulsified into droplets and then polymerization is initiated.

Liposomal formulations are typically produced by extruding or homogenizing a suspension of dissolved, hydrated lipids.[56] This suspension can then be delivered via nebulization, freeze-dried, or incorporated into larger particles. Liposomes produced in this manner have been dispersed into lactose and spray dried to produce nanoparticle-containing dry powders for pulmonary delivery.[57] Aerosolized liposomes have been used for a variety of pulmonary drug delivery applications, including local delivery of chemotherapeutics to the lung,[58] and systemic delivery of peptides.[59]

1.4.1. Spray Drying to Produce Pure Drug Nanoparticles

Spray drying is a process that uses jets of drug nanoparticles suspended in an aqueous solution of lactose or other suitable bulking agent that are forced through high pressure nozzles to produce a fine mist.[45] The aqueous or other liquid contents of the mist evaporate, leaving behind a fine powder. A new modification of spray drying, called air nebulization spray drying, uses two wedge-shaped nozzles through which compressed air passes and two through which liquid solutions pass at high velocity. The wedge-shaped nozzle acts as a fluid acceleration zone where the four streams collide at high velocity, producing a shock wave that generates fine droplets. The droplets will then descend into a column while being dried into a solid powder by heated air before being collected.

Mizoe, *et al.* have investigated this process as a means to produce nanoparticle-containing microparticles suitable for pulmonary drug delivery.[60] Briefly, pranlukast hemihydrate (PLH) was used as a water-insoluble model drug. The drug solution was prepared by dissolving PLH in a 1:1 solution of aqueous bicarbonate and ethanol. Mannitol was dissolved in water for use as the microparticle carrier. The solutions were then passed through the liquid passages of a spray dryer, as described above, and the resulting particles were collected and analyzed. This method produced particles with a mean diameter of approximately 2 μm, which is within the respirable range. The microparticles, being mostly mannitol, were dissolved in water, reportedly leaving behind a suspension of insoluble PLH nanoparticles within the size

range of 100 and 430 nm.[60] Thus, the particles produced in this manner presumably would rapidly dissolve on the surface of the pulmonary epithelium, leaving behind drug nanoparticles that can slowly dissolve and release drug for systemic or local action.

By using a similar method, Sham, *et al.* spray dried lactose with either gelatin or poly(butyl)cyanoacrylate nanoparticles to produce particles for pulmonary delivery.[61,62] In one study, the authors produced gelatin nanoparticles by dissolving high molecular weight gelatin in water and stirring under constant heating at 40°C, and the pH was adjusted to 2.5. Acetone was added drop-wise to the solution to precipitate the nanoparticles, which were then stabilized with 25% glutaraldehyde as a cross-linking agent. The particles were then stirred for 12 hours to remove the residual solvent. Poly(butyl)cyanoacrylate nanoparticles were prepared by adding monomer drop-wise to a 0.01 N HCl solution. The solution was then stirred for 4 hours and the pH was adjusted to 5.0 with 1 N NaOH. Particle sizes were reported to be around 240 nm and 170 nm for the gelatin and poly(butyl)cyanoacrylate nanoparticles, respectively, by dynamic light scattering.[62] Particles were then spray dried using a parallel flow nozzle, which sprays solution and drying air in the same direction to atomize the drug solution. In this experiment, lactose was dissolved in water at about 7% weight per volume (5 g per 75 mL) and heated to increase the lactose solubility. The solution was then mixed with one of the two nanoparticle suspensions (1:3 volume of nanoparticle

suspension to lactose solution) and spray dried. The authors report producing powders with sizes of 2.50 μm, 2.59 μm, and 2.60 μm for pure lactose, lactose and gelatin nanoparticles, and lactose and poly(butyl)cyanoacrylate nanoparticles, respectively.[62] The inclusion of the nanoparticles in the formulations did not significantly affect the particle sizes, as determined by a Student's t-test.[62] This study demonstrates that polymeric nanoparticles can be incorporated into larger particles with suitable sizes for pulmonary delivery. The authors have also reported that these particles have applications as "cluster bombs" that can be used to deliver chemotherapeutics to lung cancer cells. In another study, the authors demonstrated that polymeric and liposomal nanoparticles loaded with doxorubicin and spray dried with lactose in the manner described above were more effective at killing chemo-sensitive (H460 cell line) and chemo-resistant (A549 cell line) lung cancer cells than pure drug and unloaded nanoparticles.[63]

In another study, Yamamoto, *et al.* combined an emulsion/solvent evaporation technique with a modified form of spray drying to produce microparticles suitable for inhalation.[64] Briefly, PLGA and the drug 6-coumarin were dissolved in an ethanol and acetone mixture and injected into an aqueous polyvinylalcohol (PVA) solution while being stirred at 400 rpm, producing particles approximately 250 nm in diameter after evaporation of the organic phase.[64] Lyophilized powder was then suspended in water containing

dissolved mannitol and spray dried in a fluidized bed granulation system. In this process, solution is sprayed from the bottom of the reactor into the granulation chamber and the resulting mist was dried by heated air. Dried particles were then entrapped onto a backdrop filter and redispersed into the granulation chamber with a pulsed air jet. The authors hypothesize that the particles were granulated by the coalescence of wet and dry particle collisions within the granulation chamber.[64] The authors report an aerodynamic diameter of the resulting particles between 1 and 10 μm, as measured by cascade impaction.[64]

Spray drying has also been used to create powders with effervescent properties for more active release in the lungs. Ely, *et al.* prepared poly(butyl)cyanoacrylate nanoparticles as a model particle.[65] The resulting nanoparticles were then suspended in a aqueous solution containing citric acid, sodium carbonate, polyethylene glycol 6000 and L-leucine.[65] The suspension was then spray dried to produce particles with an aerodynamic diameter of 2.17 μm. The authors reported that, when dissolved in water, the effervescent particles demonstrated more active release of nanoparticles as compared to lactose control particles, as determined by the large (~30 μm) nanoparticle-filled bubbles that were observed with the effervescent powders.[65] This type of formulation could decrease the residence time of the microparticles in the lung tissue, potentially decreasing the chance of being phagocytized by alveolar macrophages, thus possibly enhancing the

bioavailability of the drug. Further research should be done in this area to evaluate the potential benefits of this type of formulation.

Spray freeze-drying into liquid (SFL) is a variation of spray drying that atomizes drug and excipient streams into a cryogenic liquid to produce frozen particles. The particles are then collected and lyophilized to obtain a dry powder. [45,46] Hu, *et al.* demonstrated that this process could be used to produce danazol nanoparticle-containing microparticles with fast dissolution rates.[45] In another study, Purvis, *et al.* showed that this process can be used to engineer intraconazole-containing particles suitable for pulmonary delivery, using polyvinylpyrrolidone as the excipient.[46] After spray freeze-drying, the particles were dispersed in saline solution, then nebulized and administered to mice as a prophylactic treatment against *Aspergillus flavus* infection. Mice treated with nebulized SFL particles, as well as an evaporative precipitation of aqueous intraconazole as a control, had a greater chance of survival than mice that were not inoculated.[46] The authors did not report a significant difference in prophylactic ability between the nebulized particles and the aqueous intraconazole control.

1.4.2. Supercritical Fluid Extraction to Produce Pure Drug Nanoparticles

Supercritical fluid extraction is a chemical process that uses a supercritical fluid (often $scCO_2$) to extract solvent or other impurities from a suspension. Supercritical carbon dioxide usage has gained popularity recently because it

is an environmentally benign solvent that can be harmlessly vented into the atmosphere.[66] Additionally, because carbon dioxide is a gas at ambient temperature and pressure, it can simply be flash vaporized at atmospheric pressure, leaving behind any extracted solvent and impurities. This eliminates the extraction medium stripping step from the process, making it more economical and less energy intensive than traditional extraction and stripping processes. Recently, Chattopadhyay, *et al.* used a continuous supercritical carbon dioxide extraction process to produce solid lipid nanoparticle suspensions for pulmonary delivery.[44] In this process, supercritical carbon dioxide was used to extract organic solvent from an oil in water emulsion containing one of three lipids (tripalmitin, tristearin, or gelucire 50/13), and one of two model drugs (indomethacin, or ketoprofen).[44] One of the aforementioned lipids and drugs was dissolved in chloroform with a soy lecithin surfactant, then dispersed into an aqueous solution containing sodium glycocholate and homogenized under high pressure to produce the emulsion.[44] This reportedly created an emulsion with a mean droplet size ranging between 30 and 100 nm, which was introduced into an extraction column countercurrently to a stream of supercritical carbon dioxide. The $scCO_2$ extracted the organic solvent from the dispersed droplets, leaving behind solid lipid, drug-containing particles in an aqueous suspension. [44] This processing method produced nanoparticles with a volume mean diameter between 10 and 30 nm, and a drug loading efficiency between 80 and 90%

for the gelucire particles and 10% for the tripalmitin particles. Nebulized droplets were produced from the suspensions within an aerodynamic diameter range between 2 and 4 μm, which is within the respirable range.[44] Thus, supercritical fluid extraction might be an effective means to produce drug-loaded nanoparticles within a suitable size range for pulmonary delivery as a nebulized aerosol.

1.4.3. Methods to Produce Polymeric Nanoparticle Formulations

Several studies have examined different polymeric nanoparticle formulation techniques and their suitability for pulmonary drug delivery applications. These techniques generally involve polyelectrolyte complex formation,[67] double emulsion/solvent evaporation techniques,[53,55,67] or emulsion polymerization methods.[54] In polyelectrolyte complex formation, a polycation and a polyanion are dissolved (usually in water), then mixed under moderate shear to generate nanoparticles. Alternatively, a single polyionic material can be complexed with an oppositely charged drug and then gelled with an oppositely charged, multivalent salt.[52] Other studies have condensed DNA (a polyanion) with a polycation to produce nanoparticles for pulmonary gene delivery.[67] Double emulsion methods typically dissolve a polymer such as PLGA in an organic solvent, which is then emulsified under shear in aqueous solvent containing surfactants. The organic solvent is then typically evaporated, leaving a colloidal suspension of solid nanoparticles. [50,51,53,55]

Emulsion polymerization methods involve adding a liquid monomer to an aqueous solution containing surfactants and applying shear, which is followed by polymerization of the dispersed phase. For example, this technique sometimes employs (poly)cyanoacrylates, which polymerize in water, thus eliminating the need for an initiator.[54]

Grenha, *et al*.,[52] produced nanoparticles using an ionotropic gelation method. Chitosan and tripolyphosphate (TPP) were dissolved in aqueous solutions and mixed under mild stirring to spontaneously precipitate nanoparticles. Insulin was dissolved in 0.01 M NaOH solution and added to the TPP solution before being added to the chitosan solution. Particles with sizes ranging from 300 to 500 nm and with zeta potentials between +32 to +45 mV were produced in this manner. The authors found that increasing the chitosan to TPP mass ratio decreased the process yield (mass of particles produced over total mass), but increased the size and zeta potential of the particles.[52] The particles decreased in size when incubated in a lysozyme solution (due to hydrolysis of the β-(1-4) glycosidic linkages in chitosan), suggesting that they will degrade in the pulmonary epithelium and release their drug contents. These particles were then incorporated into microparticles by incubating them in lactose and mannitol excipient solutions and spray drying. This process produced microparticles with aerodynamic diameters between 2 and 3 μm, which are suitable for pulmonary delivery.[52] The microparticles rapidly dissolved in aqueous solution, leaving behind a

suspension of nanoparticles. The authors conclude that these particles can be used to effectively deliver therapeutic macromolecules to the lungs and promote pulmonary absorption.[52]

In a recent study,[68] the authors demonstrate that the same chitosan/TPP nanoparticles can be incorporated into phospholipid microparticles using a lipid film evaporation method. Briefly, the phospholipids dipalmitoylphosphatidylcholine (DPPC) or a mixture of DPPC and dimyristoylphosphatidyl glycerol (DMPG) (10:1 molar ratio) was dissolved in chloroform and evaporated. The resulting lipid film was then hydrated with a suspension of nanoparticles.[68] The resulting microparticles were reported to have an aerodynamic diameter between 2.1 and 2.7 μm, making them suitable for delivery to the peripheral lung.[68]

Another method of nanoparticle synthesis described by Dailey, *et al.* capitalizes on both ionotropic complexation and solvent displacement techniques.[51] The authors developed a novel branched polyester, diethylaminopropyl amine-poly(vinyl alcohol)-grafted-poly(lactide-co-glycolyde) (DEAPA-PVAL-g-PLGA), which contains a degradable PLGA backbone and a cationic tertiary amine group. The authors hypothesize that the additional hydrophilic derivatization can increase the degradation rate of the polymer by increasing the degree of water saturation.[51] To formulate the nanoparticles, the DEAP-PVAL-g-PLGA polymer was dissolved in acetone and injected into water containing a buffer and carboxymethyl cellulose

(CMC); a polyanion. The resulting suspension was then stirred under reduced pressure to remove the organic solvent. The authors found that the ratio of polymers had a great impact on both the particle size and the zeta potential. As the concentration of CMC increased, the zeta potential decreased, and as the zeta potential approached neutrality, the particle size increased. Additionally, as the concentration of the CMC increased further, the zeta potential continued to decrease, and the particle size began to decrease. The authors suggest that this is most likely due to increased particle agglomeration as the surface charge approaches neutrality, thereby diminishing Coulombic forces between particles.[51] The authors found that the increased hydrophilicity of the nanoparticles increased the rate of degradation as compared to pure PLGA particles. They also observed that the more neutral the zeta potential of the particles, the less rapidly they degraded over time, presumably due to an increase in lipophilicity.[51] This study also reported an increased stability in negatively charged particles after nebulization.[51] In a later study, the authors showed preliminary evidence that particles produced in the same manner possess a decreased inflammatory potential in the lung compared to non-degradable, lipophilic polystyrene particles of similar size.[50] Thus, particles produced in this manner may have potential as a pulmonary drug delivery vehicle that degrades relatively rapidly and does not cause inflammation.

Another study utilized both polyionic complexation and solvent displacement to develop a PLGA polyethylenimine (PEI) nanoparticle complexed with DNA for gene delivery to the pulmonary epithelium.[67] Bivas-Benita, *et al.* dissolved PLGA and PEI in dichloromethane, and added Tween-80 and acetone. This solution was injected into an aqueous phase containing Poloxomer-188 as a surfactant, and stirred slowly to evaporate the organic phase.[67] The resulting particles were filtered, and then complexed with DNA. To achieve this, a nanoparticle suspension was added to a DNA solution and vortexed. Particles were created at varying PLGA-PEI ratios and PEI-DNA ratios.[67] This method consistently produced particles with sizes between 207 and 231 nm, regardless of the PLGA-PEI and PEI-DNA ratios used. The authors also observed that the zeta potential was not affected by the PLGA-PLA ratio, but was dependent on the PEI-DNA ratio.[67]

Shi, *et al.* have shown that oppositely charged nanoparticles can be assembled to form larger, low density microstructures suitable for pulmonary delivery.[53] In this study, PLGA nanoparticles were produced using a solvent evaporation procedure. Briefly, PLGA was dissolved in acetone and methanol, then injected at a controlled rate into an aqueous solution containing either dissolved poly(ethylene-maleic anhydride) (PEMA) or poly(N-vinyl formamide) (PVAm) under homogenization. The residual acetone was then evaporated, and the remaining particles were collected. Particles produced in the PEMA solution were around 300 nm in diameter

with a zeta potential of around -50 mV, and particles produced in PVAm solution were slightly larger at around 500 nm and with a zeta potential of around +30 mV. The difference in zeta potential was due to the charges of the coating polymers, PEMA being a polyanion and PVAm a polycation.[53] A suspension of the PVAm-coated particles were then injected into a suspension of PEMA-coated particles under homogenization to destabilize the colloid and cause particle flocculation; induced by the ionic interactions between particles in suspension. The authors found that lyophilized samples of the nanoparticle flocculates has aerodynamic diameters between 2 and 4 μm, making them ideally sized for pulmonary delivery.[53] Additionally, SEM images showed that the nanoparticle flocculates had irregular geometries and were largely porous structures, which would increase their flowability in a dry powder inhaler.[53]

PLGA is often favored as a polymeric excipient for drug delivery because of its biodegradability and biocompatibility. In a study by Pandey, *et al.*, PLGA nanoparticles were prepared using a multiple emulsion/solvent evaporation technique similar to those previously described.[55] The authors produced nanoparticles containing rifampicin, isoniazid, or pyrazinamide, which are used to treat tuberculosis. Briefly, aqueous drug solution was emulsified in a dichloromethane solution containing PLGA polymer via probe sonication. The emulsion was then added to an aqueous solution containing polyvinyl alcohol and sonicated. The resulting solution was then stirred

overnight to remove the organic phase, and particles were collected via centrifugation.[55] This technique produced particles with sizes ranging between 180 and 300 nm and a drug encapsulation efficiency between 50 and 70%, depending on the drug used. The particle suspensions were then nebulized, reportedly producing droplets between 1.1 and 2.1 μm, which is within the respirable range.[55]

Another method of nanoparticle synthesis is emulsion polymerization, where a monomer emulsion is prepared and polymerized as nanoparticles. Studies suggest that cyanoacrylates are popular monomers for this method, most likely because of their water-initiated polymerization process. In a study by Zhang, *et al.*, insulin-loaded poly(butyl)cyanoacrylate nanoparticles were produced by injecting α-butyl-cyanoacrylate drop wise into an aqueous solution containing Dextran 70.[54] After 1 hour of agitation, insulin solution was added and agitation was continued for another 2 hours. This processing method reportedly produced particles with sizes ranging between 160 and 400 nm and an insulin association ratio of 79.1%.[54] Interestingly, the *in vitro* release profiles of the insulin nanoparticles were biphasic, consisting of a burst phase followed by a zero-order release phase. The authors hypothesize that this is most likely due to the rapid dissolution of insulin adsorbed to the surface of the particles, followed by sustained release of insulin contained within the particles.[54] The authors also observed a prolonged hypoglycemic effect in rats when the particles were administered

intratracheally as compared to an insulin solution delivered via subcutaneous bolus.[54] This study suggests that insulin, and possibly other small peptide drugs, can be adsorbed onto nanoparticles and delivered to the lungs to produce a range of release profiles.

1.4.4. Methods to Produce Liposomal Nanoparticle Formulations

Liposomal nanoparticles have been investigated as potential drug carriers to the lung. Many lipids commonly used in liposome preparation are already abundantly present in the lungs, thus they should be well tolerated by the lungs.[56] Additionally, the great diversity of amphiphilic molecules available for constructing liposomes provides enormous versatility in particle size and physical parameters, making them highly attractive for creating a variety of drug delivery vehicles.[69] A possible concern is that lipids themselves modify the surface tension of the lungs and have themselves been used as therapeutics.[70,71]

In general, liposomes are interesting potential drug delivery vehicles because of their stability in suspension. For instance, upon dilution or changes in ionic strength that may be encountered during administration, liposomes will theoretically remain intact. This is because liposomal formulations are not thermodynamically equilibrated systems, but are kinetically trapped systems that are unable to respond to thermodynamic perturbations.[69] This property might make them suitable for formulations that

require long-term storage as liquid suspensions, such as those used in nebulization.

Liposomes have also been investigated as potential targeting agents for alveolar macrophages,[72,73] partially because macrophages are associated with lung surfactant metabolism.[72] Studies have also shown that mannosylation of liposomal nanoparticles[72,73] and particle size[72] influence the uptake of liposomes by macrophages. The studies demonstrate that liposomal uptake increases as particle size increases over a range of 100 to 2000 nm,[72] and as the degree of mannosylation increases on the surface of the particles.[72,73] The authors suggest that this is likely due to the fact that mannose receptors are exclusively expressed on the surface of alveolar macrophages.[73]

In addition to their ability to target alveolar macrophages, liposomes have been investigated because of their surface chemistries, which can be modified with chemical linkers to produce liposome agglomerates.[56] Bhavane, *et al.* developed a novel type of drug delivery vehicle composed of liposomal nanoparticles covalently linked by enzymatically-labile spacers. In this study, liposomes were prepared by extruding a suspension of dissolved, hydrated lipids.[56] The lipid mixture included dipalmitoylphosphatidylcholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine-poly(ethylene glycol)-amine (DSPE-PEG-NH_2), which contains a amine group suitable for conjugation.[56] This process reportedly produced liposomes

between 80 and 195 nm in size.[56] Liposomes were loaded with ciprofloxacin with 90% efficiency.[56] Agglomeration was induced using dimethyl 3,3'-dithiobispropionimidate 2HCl (DTBP), which is a homobifunctional imidoester capable of reacting with primary amines and also contains thiol-cleavable disulfide bonds, making it enzymatically labile.[56] The authors found that at low pH, smaller agglomerates were produced due to the restricted activity of the linker. The agglomerated liposomes demonstrated slower release kinetics than the unagglomerated liposomes, and a burst effect was observed at time points when dithiothreitol (a disulfide bond reducing agent) was added to the dissolution medium. Upon nebulization, the agglomerated particles were reported to retain the encapsulated drug and nebulized droplets had aerodynamic diameters between 1 and 5 μm, putting them within the respirable range.[56] This study reports that controlled agglomeration of liposomal nanoparticles can be achieved, and that these particles can be nebulized into the respirable range.

Liposomal formulations have also been investigated in potential dry powder formulations. Chougle, *et al.* loaded nano-sized liposomes with tacrolimus, an immunosuppressant often used to prevent rejection of allotropic lung transplants. The authors used a thin film evaporation technique, which included dissolving the drug and lipids (hydrogenated phosphatidylcholine and cholesterol) in methanol and chloroform, followed by solvent evaporation.[57] The liposomes were then hydrated using PBS, and the

resulting suspension was passed through a high-pressure homogenizer to produce particles approximately 140 nm in diameter. The nanoparticles were then suspended in an aqueous solution containing either lactose, sucrose, or trehalose and L-leucine and spray dried to produce microparticles with an aerodynamic diameter of approximately 2.2 μm that demonstrated good aerosolization properties when administered from a DPI.[57] This study reportedly demonstrates that liposomal nanoparticles have potential use in dry powder pulmonary formulations.

1.5. Toxicity Considerations of Nanoparticle Formulations

A major concern with nanoparticle therapeutics is the unforeseen negative health impact that nanoparticles may have. Studies in rodents have shown that intratracheally-administered carbon nanoparticles accelerate vascular thrombosis.[74] Additionally, it has also been demonstrated that inhaled iridium particles may migrate from the lung to the systemic circulation, which may have detrimental vascular effects,[75,76] and inhaled carbon nanoparticles have been shown to migrate to the brain, although their CNS toxicity remains to be determined.[77] Conversely, a recent study has shown that metallic nanoparticles are no less cytotoxic than metallic microparticles, suggesting that the small size of nanoparticles may not be the most important factor affecting their toxicity.[78] This notwithstanding, the toxicity of nanoparticles is a legitimate concern and should be thoroughly investigated.

In addition to the possible inherent toxic effects of nanoparticles, some materials used to formulate nanoparticles may have toxic effects and therefore may not be viable for developing therapeutic products. For example, the toxicity of polycyanoacrylates has been demonstrated by Brzoska, *et al.* The authors prepared poly(butyl)cyanoacrylate and poly(hexyl)cyanoacrylate nanoparticles in a method similar to that described by Zhang, *et al.*,[54] using either Dextran 70 stabilizer or poloxamer 188.[79] They determined that both types of nanoparticles caused an increase in lactate dehydrogenase (LDH) activity in human pulmonary epithelial cells. The degree of toxicity was greater for the poly(butyl)cyanoacrylate and independent of the stabilizer used, which stands to reason because shorter chain polycyanoacrylates have been associated with higher cytotoxicity.[79] The degree of toxicity also increased with increasing nanoparticle concentration,[79] most likely due to the subsequent increase in polycyanoacrylate concentration. Polyethyleneimine (PEI) has also demonstrated cytotoxicity in lung cells.[80] Bivas-Benita, *et al.* showed that when PEI-DNA complexes were used to deliver DNA to lung epithelial cells, cell viability decreased as the PEI-DNA ratio increased.[67] Despite this, Dailey, *et al.* have shown that PLGA nanoparticles induce less inflammation than polystyrene particles of similar size when delivered to the lungs.[50] Based on this observation, nanoparticle toxicity in the lungs may be more dependent on material choice than particle size. Therefore, there may

be alternative polymers that can be investigated for use in pulmonary nanoparticle drug formulations that could mitigate toxicity.

1.6. Future Directions

Nanoparticle drug formulations offer many advantages over traditional aerosol powders and liquid pulmonary dose formulations. For example, the bioavailability of poorly water-soluble drugs can be greatly enhanced by the large surface area of drug nanoparticle formulations. Additionally, nanoparticles can be formulated in such a way to offer enhanced control over the morphology of dry powder drug formulations and the ability to produce structures with both a low-density microstructure for delivery to the deep lung and nanostructure for enhanced dissolution and bioavailability.

The literature suggests many different formulation approaches for drugs that use a variety of excipients to fabricate nanoparticles or nanoparticle complexes suitable for pulmonary delivery. Many chemical processing techniques such as supercritical fluid extraction and spray drying have been successfully used for therapeutic nanoparticle processing. Additionally, polyelectrolyte complexation, double emulsion and solvent evaporation, emulsion polymerization, and liposomal loading offer a range of formulation options. This diverse array of techniques has demonstrated the ability to effectively produce nanoparticles with a high degree of control over particle properties; however residual solvents, cytotoxic excipients, low drug

loading efficiencies and scale-up issues might limit their commercial applications. With the perfection of pulmonary nanoparticle drug formulations, the lungs may become a preferred route of drug delivery for many local and systemic therapeutic interventions.

2. Design of a Nanoparticle-Based Pulmonary Insulin Formulation

Diabetes mellitus is a set of diseases characterized by defects in insulin utilization, either from autoimmune destruction of insulin-producing cells (Type I) or insulin resistance (Type II). As of 2005, 20.8 million people in the United States (7.0% of the population) suffered from diabetes, and it was the sixth leading cause of death due to the many complications associated with this disease, such as pulmonary hypertension and ischemia.[81] Current treatment methods involve regular injections of insulin, which can be both painful and inconvenient, thus often leading to low patient compliance.[82]

In order to overcome this problem, other routes of insulin administration have been investigated. Inhaled aerosols have been shown to be an effective means to treat local diseases of the lung.[83] Additionally, the large surface area of the lungs (~140 m^2) and their ready access to systemic circulation makes them a possible candidate for noninvasive, systemic drug delivery. This is particularly good for macromolecular drugs such as peptides, proteins, and DNA.[83-85]

Sufficient deposition of aerosol particles in the peripheral lung requires precise control over particle size and density, which greatly affect the region of deposition in the lungs.[7,84-88] Particles possessing an aerodynamic diameter in the range of 1-5 μm are required for suitable terminal bronchiole and alveolar deposition,[7,86,88,89] as a means to access systemic bioavailability.[87] Low-density particles are currently being developed as a means to deliver drugs to the distal regions of the lungs.[85,87,90,91] These particles possess large geometric diameters, but smaller aerodynamic diameters due to their low density, as described by the following equation,[7,53,86]

$$d_{aero} = d_{geo}\left[\frac{\left(\rho / \rho_{ref}\right)}{\gamma}\right]^{0.5} \quad [1]$$

Where ρ_{ref} is a reference density (for example 1 g/cm^3) and γ is the shape factor (equal to 1 for a sphere).

Studies have shown that insulin delivered via the pulmonary route is well tolerated and effective in treating patients with Type I diabetes.[89,91-97] Currently, there are several inhaled insulin delivery systems in development. Of these, those employing the Technosphere (Mannkind Corporation) and Spiros technologies (Dura Pharmaceuticals) are dry powder formulations.[92] The Exubera formulation (Nektar Therapeutics and Pfizer) and the

formulation using AIR technology (Alkermes and Eli Lilly) are other examples of dry powder formulations that were either FDA approved and discontinued (Exubera), or terminated during Phase III clinical trials (AIR). One potential drawback to these formulations is that they contain excipients, which aid in manufacturing and aerosol performance, but may have unforeseen negative impacts on the long-term respiratory health of the patient.[82,95] Of particular concern are penetration enhancers, such as polyoxyethylene 9 lauryl ether and sodium glycocholate, which have been shown to induce acute inflammation in the lung.[98] It may therefore be desirable to create an inhalable form of insulin that does not contain excipients so as to avoid any potential complications that might arise. Additionally, most of the current technologies use a spray drying technique to produce particles, which can subject the insulin to air/water interfaces, high temperatures, and other conditions that can cause the protein to denature.

Here, a dry powder Zn-insulin formulation possessing appropriate microstructure to reach the deep lung that is processed without excipients has been developed. Factors such as pH and insulin concentration were shown to have an affect on seed nanoparticle size. Circular dichroism (CD) and solid-state nuclear magnetic resonance (ssNMR) were used to show that irreversible secondary structure and crystallinity changes of the insulin did not occur as a result of processing. It has been demonstrated that excipient-free, insulin microparticles that are suitably sized for pulmonary delivery and have

a high dissolution velocity can be produced with minimal processing steps. This development may help future researchers develop novel pulmonary delivery systems for the treatment of diabetes.

2.1. Materials and Methods

2.1.1. Materials

Lyophilized insulin powder from bovine pancreas (0.5% zinc content) and phosphate buffered saline premix (PBS) were purchased from Sigma (St. Louis, MO). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification.

2.1.2. Fabrication of Insulin Nanoparticles

Approximately 100 mg of insulin stock powder were dissolved in 15 mL of 0.01 N HCl solution. The solution was then titrated drop-wise to a pH just below the isoelectric point (pI) of the native protein (5.3) with 0.01 N NaOH solution, at which point the solution became colloidal without fully precipitating. The mean geometric diameters and polydispersities of the nanoparticle suspension were measured using dynamic light scattering (Brookhaven Instruments Zeta Potential Analyzer, Holtsville, NY). Nanoparticles were diluted in deionized H_2O (~100X) and three, 1 minute measurements were obtained at 25°C for each sample. Mean size and polydispersity were determined by the method of cumulants.[99] The same instrument was used to

determine the zeta potential (ζ) of the nanoparticles in 1 mM potassium chloride solution. Three runs of 15 cycles were acquired, and the mean zeta potential was recorded. Some samples were frozen at -80°C and lyophilized using a Labconco bench top lyophilizer (Kansas City, MO) for further analysis.

A range of pH values near the pI of the native protein were determined in which the nanoparticle colloid was preserved. Particle sizes and zeta potentials were measured for each sample. Nanoparticle samples within this pH range (from 4.92 to 5.09) were centrifuged at 13,000 rpm for 10 minutes and the supernatant concentration of insulin was analyzed using UV absorbance spectroscopy (Agilent 8453). All pH values were measured in triplicate. The measured concentration was used to calculate the mass of insulin in the pellet from the original insulin mass and total volume.

2.1.3. Agglomeration of Insulin Microparticles

Aliquots of insulin nanoparticle suspensions (5 mL) were added to 15 mL of ethanol and stirred for ~36 hours at 300 rpm under a fume hood. Nanoparticles with diameters of approximately 200 nm were selected for this step. The geometric diameters of the insulin microparticles were measured using a Coultier Multisizer™ 3 (Beckman Coultier, Fullerton, CA). Samples were then frozen at -80 °C and lyophilized for further analysis.

2.1.4. Characterization of Aerosol Properties

The aerodynamic diameters of the lyophilized powders were determined using an Aerosizer LD (Amherst Process Instruments Inc.). Data were collected over ~70 seconds under high shear force (~6 psi) using a 700 μm aperature.

2.1.5. Characterization of Particle Morphology

The size and morphology of lyophilized samples were evaluated using a LEO 1550 field emission scanning electron microscope (SEM). All samples were sputter-coated with gold for 30 seconds prior to imaging.

2.1.6. Conformational Stability of Processed Insulin

Post-processing secondary structural changes in samples were analyzed by dissolving particles in 0.01 N HCl solution and analyzing using circular dichroism spectroscopy (CD; Jasco J-810, Easton, MD) to determine conformational differences between processed and unprocessed insulin, as well as thermal stability differences between groups. CD spectra were acquired in three accumulations from 260-195 nm with a scanning speed of 50 nm/min and 1.0 nm resolution. Thermal stability was determined at a wavelength of 210 nm from 10-80 °C with a scanning speed of 15 °C/hr. Thermal stability spectra were acquired in triplicate. Insulin concentration in prepared solutions was determined by UV absorbance spectroscopy.

2.1.7. Crystallinity of Processed Insulin

2.1.7.1. NMR

Spectra were collected using a Tecmag Apollo spectrometer operating at 300 MHz using ramped amplitude cross-polarization (RAMP),[100] magic-angle spinning (MAS),[101] and SPINAL-64 decoupling.[102] Samples were packed in 4 mm o.d. zirconia rotors using Teflon® encaps, and spun at 8 kHz in a ChemagneticsTM Triple-Resonance HXY CP/MAS NMR probe configured to run in double-resonance mode using the H and X channels, and fitted with a 4 mm spin module from Revolution NMR. All spectra are the sum of 120,000 transients collected using a 1.5 s pulse delay, a contact time of 2 ms, and a 1H 90° pulse width of 2.3 μs. The free induction decays consisted of 256 points with a dwell time of 33.3 μs. The spectra were externally referenced to tetramethylsilane using the methyl peak of 3-methylglutaric acid at 18.84 ppm.[103]

2.1.7.2 HPLC

The crystalline insulin content of the materials was determined using the method in the insulin zinc suspension monograph of the 2005 U.S. Pharmacopoeia National Formulary, with minor modifications. Buffered acetone TS was produced by dissolving 8.15 g of sodium acetate and 42 g of sodium chloride in 100 mL of water, to which 68 mL of 0.1 N hydrochloric acid and 150 mL of acetone were added, the mixture was then diluted with water

to make 500 mL. Approximately 0.5 mg of insulin was placed in a 1.5 mL microcentrifuge tube and 33.3 µL of a 1:2 mixture of water and buffered acetone TS was added to the tube to extract any amorphous insulin. The sample was immediately centrifuged at 13,000 rpm for one minute, the supernatant was decanted, and the extraction was repeated. Additionally, ~0.5 mg of insulin was placed in another microcentrifuge tube to be used as a control. Both insulin samples were each dissolved in 33.3 µL of 0.01 N hydrochloric acid and analyzed by HPLC, with each sample being prepared in triplicate.

The HPLC was a Shimadzu system that consisted of an SCL-10A system controller, LC-10AT liquid chromatography pump, SIL-10A auto injector with a sample cooler, and SPD-10A UV-VIS detector with instrument control and data analysis performed through CLASS-VP software. Aqueous mobile phase was prepared by dissolving 28.4 g of anhydrous sodium sulfate in 1000 mL of water, to which 2.7 mL of phosphoric acid was added and the pH was adjusted to 2.3 with ethanolamine. The aqueous mobile phase was then mixed 74:26 with acetonitrile. The separation was performed on a 4.6 x 250 mm Symmetry® C18 column from Waters that was maintained at 40°C. Samples were maintained at 5°C and 20 µL were injected for analysis, with a mobile phase flow rate of 1 mL/min and the detector set to 215 nm. Peak areas were normalized to the mass of insulin used to prepare the sample and the percent crystalline insulin was calculated with the following equation:

$$\%Crystalline = \frac{SamplePeak}{ControlPeak} \times 100 \quad [2]$$

2.1.8. Dissolution of Insulin Particles

Approximately 6 mg of each insulin particle sample was suspended in PBS (pH 7.4). The solution was placed in a 100,000 Dalton biotech grade cellulose ester dialysis tube (Spectrum Labs, Rancho Dominiguez, CA) and placed in PBS solution to a final volume of 45 mL. All samples were incubated at 37°C and shaken at 50 rpm on a shaker table. 1 mL aliquots were taken at various time points up to 8 hours from the bulk solution and replaced with 1 mL of fresh PBS. The insulin concentration was measured using a Coomassie Plus colorimetric protein quantification assay (Thermo Fisher Scientific, Waltham, MA). A calibration curve was used to correlate the insulin concentration with the measured absorbance, with insulin concentrations ranging between 1 and 25 μg/mL being used as the standard. Dissolved mass was calculated from the measured concentration, and was then normalized to the total loaded mass to determine the percent dissolved. All experiments were performed in triplicate. Analysis of variance (ANOVA) was used to determine statistically significant differences between groups ($p < 0.05$). Comparisons among groups were done using a Fisher's *F*-test.

2.1.9. Estimation of Bulk Powder Density

The bulk density of the dry powder was estimated using a micro-tap test approach, as defined in the U.S. Pharmacopoeia National Formulary, with slight modifications. Briefly, dry powder samples (unprocessed insulin, nanoparticles, and microparticles) were added to pre-weighed microcentrifuge tubes, and the tubes were weighed again to determine the mass of powder. The tubes were then tapped thirty times on the lab bench to compress the powder. The volume of the powder was approximated by comparing the height of the compressed powder to that of a volume of water in an identical pre-weighed microcentrifuge tube. The tube containing the water was then weighed to determine the volume of water (assuming a density of 1 g/mL). The powder density was calculated by dividing the mass of powder by the volume of water. All samples were analyzed in triplicate. Analysis of variance (ANOVA) was used to determine statistically significant differences between groups ($p < 0.05$). Comparisons among groups were done using a Fisher's *F*-test.

2.2. Results

2.2.1. Characterization of Insulin Nanoparticles

Zn insulin nanoparticles were created by titrating dissolved insulin to the pI of the native protein, which resulted in a colloidal suspension of nanoparticles. Particle sizes and zeta potentials were analyzed over a pH range of 4.92 to 5.09, and ranged from 292 nm to 593 nm (Table I). Zeta potentials ranged

from 10.8 mV to 18.9 mV. Neither particle sizes nor zeta potentials correlated strongly with the pH of the solution.

Table I. Characteristics of nanoparticles at various pH values.

pH	Diameter (nm)	Polydisperity	ζ-Potential (mV)
4.92	293 ± 42	0.38 ± 0.02	10.86 ± 2.4
4.97	345 ± 16	0.34 ± 0.02	15.6 ± 1.1
4.98	440 ± 58	0.36 ± 0.03	17.95 ± 1.3
5.09	592 ± 62	0.35 ± 0.01	17.62 ± 0.3

The mass fraction of insulin remaining in solution after nanoparticle precipitation was determined using UV absorbance spectroscopy. These values were used to determine the mass fraction of total insulin contained in the nanoparticles. The results suggest a positive correlation between the particle size and the total mass of the insulin nanoparticles in suspension (Figure 1).

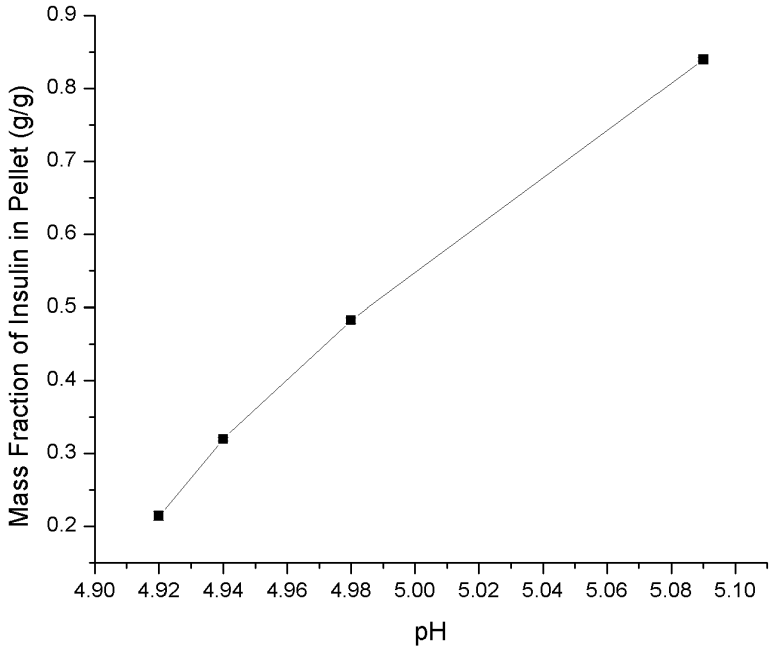

Figure 1. Mass fraction of insulin in pellet vs. pH. Each value represents mean ± S.D. of three experiments.

2.2.2. Characterization of Insulin Microparticles

Insulin microparticles were produced from insulin nanoparticle suspensions through solvent displacement. This was achieved by adding aliquots of insulin nanoparticle suspension to ethanol and stirring overnight. The geometric diameter of the insulin microparticles was determined to be 3.4 ± 1.4 μm. No correlation was determined to exist between insulin nanoparticle size and microparticle size. SEM imaging revealed differences in the morphology of the unprocessed insulin and the insulin microparticles (Figure 2). The unprocessed insulin agglomerates appear to have a more regular structure, while the microparticles have more of a leaf-like morphology. This

leaf-like shape could aid in the aerosolizability of the insulin microparticles, and would suggest a shape factor (as defined in Equation 1) of less than 1.

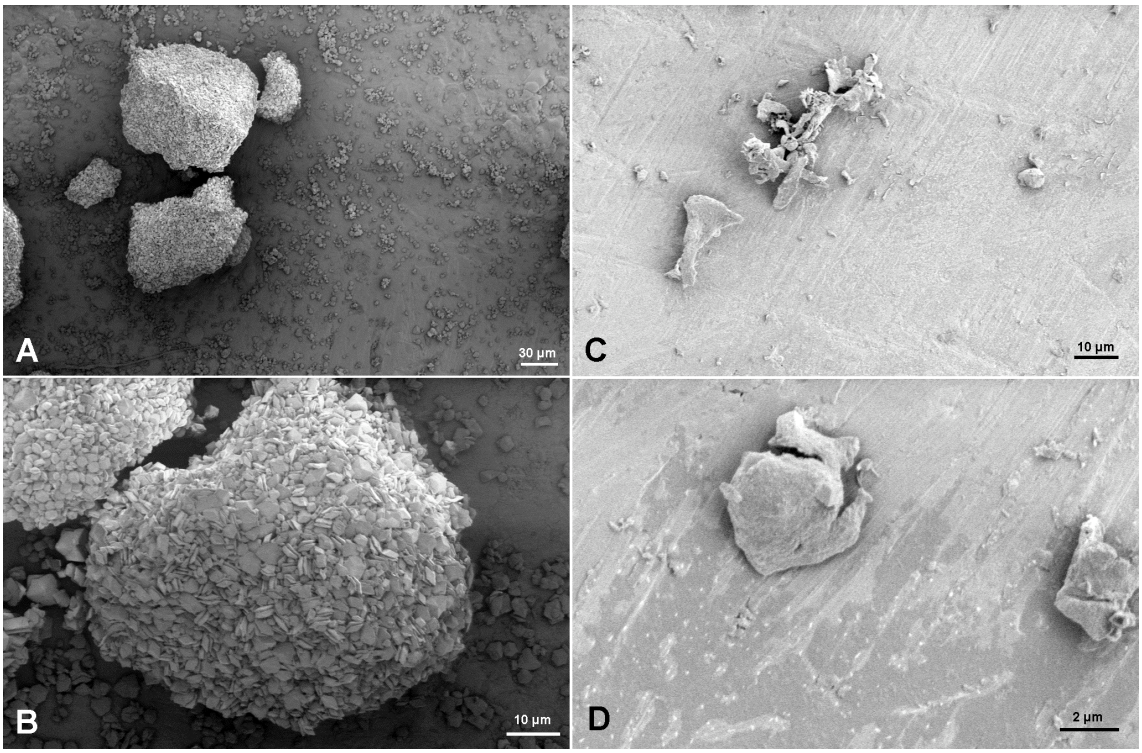

Figure 2. SEM micrographs of insulin particles; (A) and (B) are unprocessed insulin particles (scale bars 30 µm and 10 µm, respectively); (C) and (D) are insulin microparticles after processing (scale bars 10 µm and 2 µm, respectively).

2.2.3. Aerosol Properties of Insulin Particles

The aerodynamic diameters of the unprocessed insulin powder, lyophilized insulin nanoparticles, and lyophilized insulin microparticles were measured with an Aerosizer LD and are shown in Table II. The geometric diameters of the unprocessed insulin and the nanoparticles (marked with an *) were measured after resuspending lyophilized powder in aqueous solution, and the microparticles were measured in solution before lyophilization. The large aerodynamic diameter of the insulin nanoparticles is most likely due to

uncontrolled agglomeration, which probably occurred during lyophilization. The smaller aerodynamic diameter of the insulin microparticles compared to the geometric diameter of the microparticles was expected because of the lower density of the insulin microparticles (Figure 3).

Table II. Particle sizes.

Sample	d_{geo} (μm)	d_{aero} (μm)
Unprocessed*	12.0 ± 4.6	4.10 ± 1.8
Nanoparticles*	11.6 ± 9.5	3.60 ± 2.0
Microparticles	3.40 ± 1.4	2.30 ± 1.9

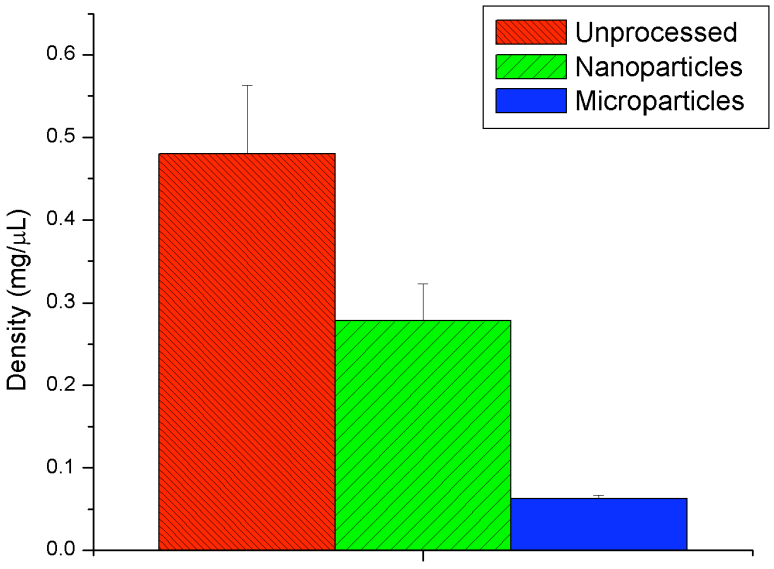

Figure 3. Tap density of insulin powders. Each bar shows mean ± S.D. of three experiments.

The size distributions of the geometric diameters are shown in Figure 4, which correspond to the mean values of the geometric diameter listed in

Table II. The highly irregular dispersion of the nanoparticle sizes is likely due to agglomeration that can occur during lyophilization.

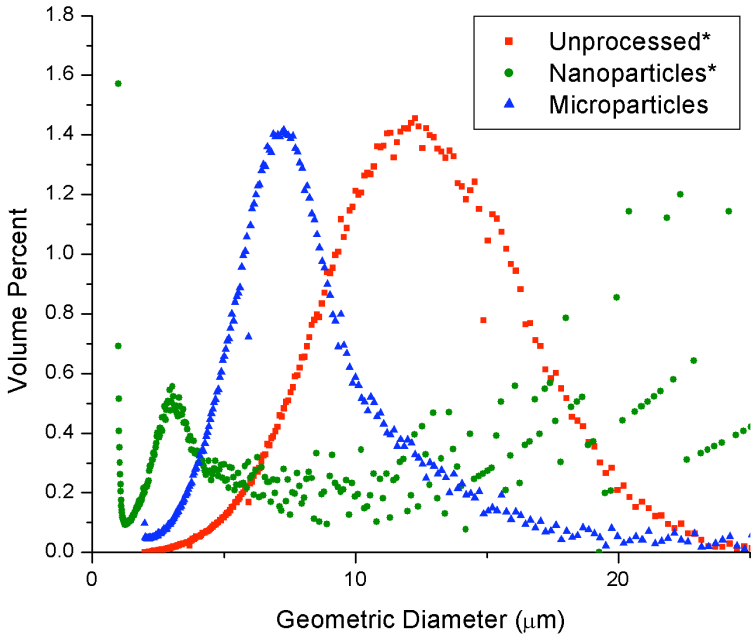

Figure 4. Size distributions of the geometric diameters of insulin powders. Samples marked with an * were lyophilized and resuspended in aqueous solution before measuring.

2.2.4. Conformational Stability of Processed Insulin

Circular dichroism (CD) was employed to analyze the secondary structure and thermal stability of processed insulin powders. Isothermal scans of dissolved, unprocessed insulin powder, dissolved nanoparticles, and dissolved microparticles reveal near-identical spectra with minima at 210 nm, suggesting that any changes in secondary structure that might occur during processing were reversible upon dissolution (Figure 5). This overlap was also

reflected in the thermal stability CD scans, which show a slight change in molar ellipticity from 10-80°C starting at about 50°C for all samples.

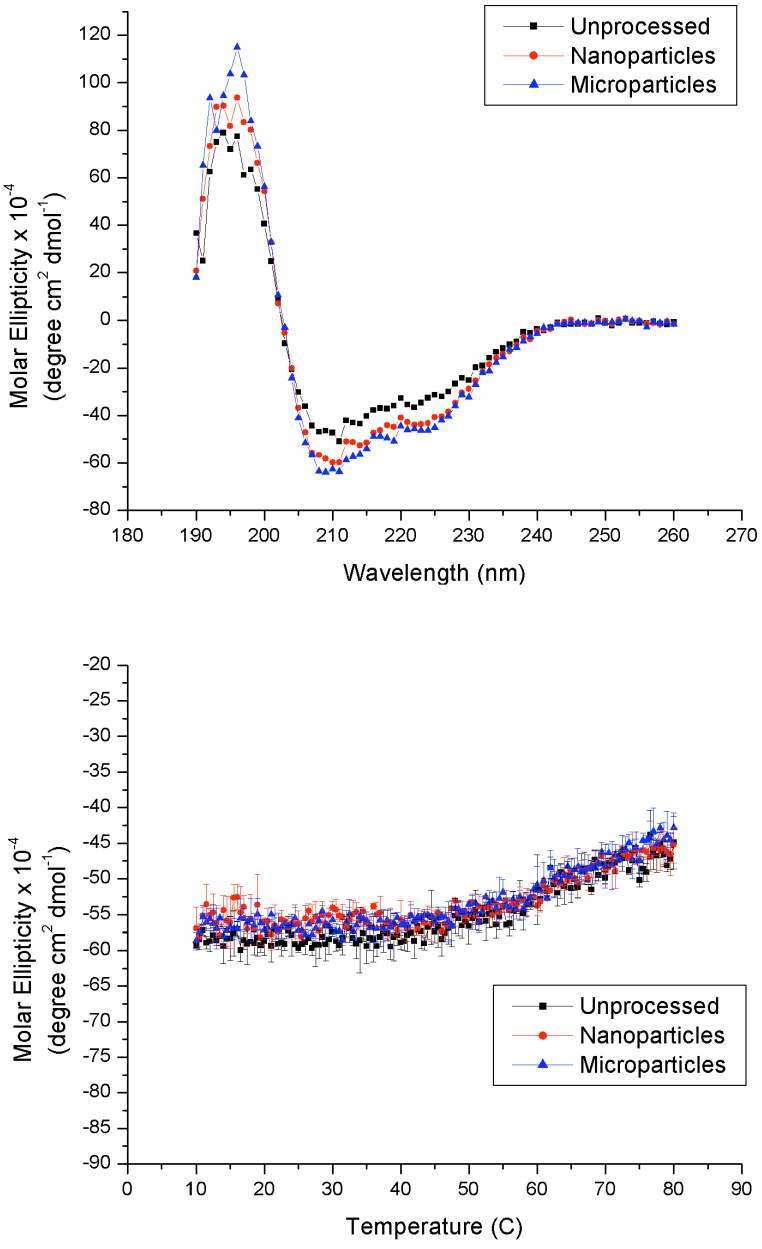

Figure 5. Circular dichroism of dissolved insulin powders. The top panel shows isothermal spectra, and the bottom panel shows variable temperature scan at a wavelength of 210 nm. Each value of the variable temperature scan represents mean ± S.D. of three experiments.

2.2.5. Crystallinity of Processed Insulin

The crystallinity of the insulin particles was examined using 13C CP/MAS NMR (Figure 6). The spectra display differences in the aliphatic region (~0 to 75 ppm), although these differences are difficult to correlate with the physical state of insulin. More obvious differences between the samples arise in the carbonyl (~175 ppm) and aromatic (~137 ppm) regions. The peak at ~137 ppm in the unprocessed insulin seems to be more narrow and better resolved than peaks at ~129 ppm. These same lines in the other samples are broader, to the point where peaks at ~129 ppm cannot be resolved. The peak at ~175 ppm in the unprocessed insulin is more narrow, with two very clear shoulders at ~180 ppm and ~173 ppm. Other samples only show one broad peak at ~175 ppm. The more defined peaks of the unprocessed insulin samples may suggest that the unprocessed insulin is more crystalline than the nanoparticles and the microparticles, however a robust interpretation of these data is difficult to ascertain.

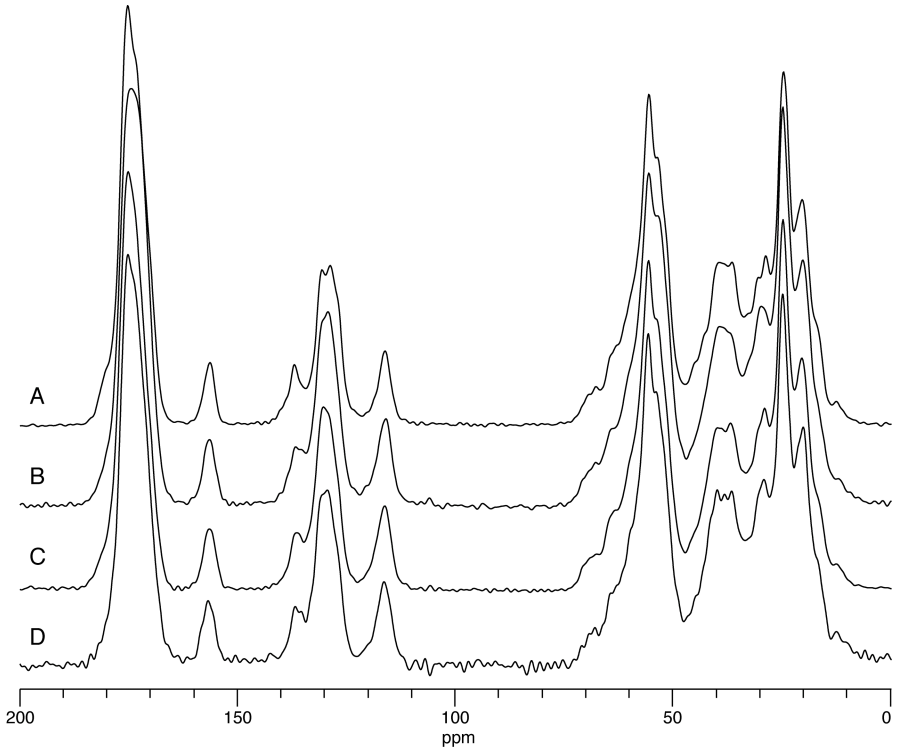

Figure 6. 13C CP/MAS NMR spectra for insulin powders; (A) Unprocessed; (B) Lyophilized insulin microparticles; (C) Lyophilized insulin nanoparticles; (D) Centrifuged and dried insulin nanoparticles.

Crystallinity of the insulin particles was also examined using the buffered acetone method described in the U.S. Pharmacopoeia National Formulary. The results suggest that the unprocessed insulin particles are between 80% and 88% crystalline, which is much greater than both the nanoparticles and microparticles, which were estimated to be between 2% and 8% crystalline, and between 17% and 24% crystalline, respectively (Figure 7).

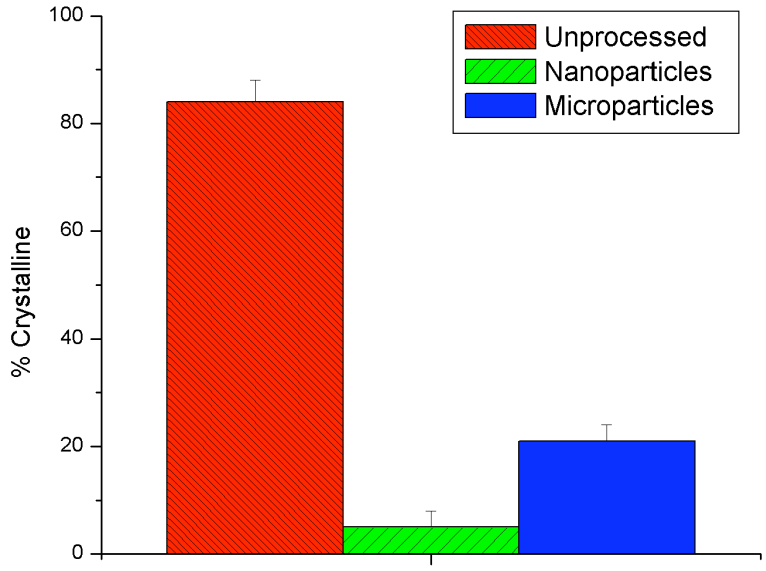

Figure 7. Percent crystallinity of insulin particles, as determined by the HPLC dissolution method described in the U.S. Pharmacopeia and National Formulary. Each bar shows mean ± S.D. of three experiments.

2.2.6. Dissolution of Insulin Particles

The concentration of insulin was measured over time in PBS solution to determine the dissolution rate of the different powders (Figure 8). The unprocessed insulin follows Higuchi dissolution kinetics,[104] and the nanoparticles and microparticles appear to show a burst dissolution phenomenon after 15 minutes. The dissolved masses of nanoparticles and microparticles were both significantly different from the dissolved mass of unprocessed powder after 15 minutes ($p = 0.0021$ and $p = 0.0054$, respectively). The dissolved masses of neither the nanoparticles nor the

microparticles were significantly different from the dissolved mass of the unprocessed insulin after 8 hours.

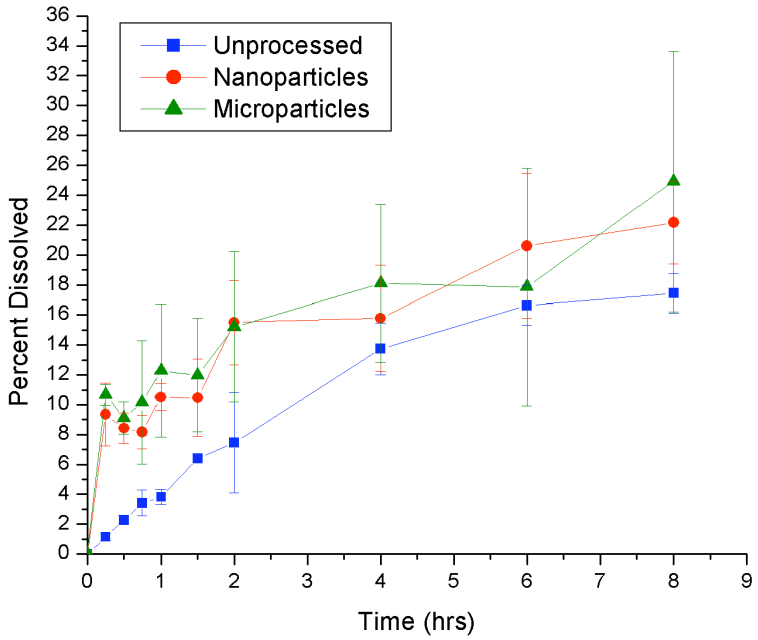

Figure 8. Dissolution of insulin powders over time. Each value represents mean ± S.D. of three experiments.

2.2.7. Bulk Powder tap Density

The tap test method was used to determine the bulk density of the insulin powders before and after processing. Density of the unprocessed insulin powder was determined to be 0.48 ± 0.08 mg/μL (Figure 3). The nanoparticle bulk density was determined to be 0.28 ± 0.04 mg/μL, and the bulk density of the insulin microparticles was determined to be 0.063 ± 0.004 mg/μL. Analysis of variance revealed a p-value of 0.00025 ($p < 0.05$), indicating a statistically significant difference between the bulk densities of each group.

2.3. Discussion

Pure insulin microparticles with sizes within the respirable range were produced from the solvent-induced agglomeration of insulin nanoparticles. Nanoparticles were produced using titration and were shown to have a strong correlation between pH and particle size (Figure 1). Microparticles were then produced using ethanol to displace the aqueous solvent and induce nanoparticle agglomeration. The proposed mechanism for this agglomeration is a combination of decreased electrostatic interactions between nanoparticles due to the addition of the organic phase, and the deposition of dissolved insulin onto the surface of the nanoparticles, forming microparticles with a leaf-like morphology. Alternatively, it might be possible that the addition of the organic phase caused a partial dissolving of the nanoparticles, thereby causing their surfaces to fuse together.

The sizes of the microparticles were independent of the size of the nanoparticles used, and had a mean aerodynamic diameter that was roughly between 0.42 μm and 4.3 μm. This range of particle sizes is similar to other dry powder insulin formulations, such as Exubera (3.5 μm),[96] and a formulation based on the Spiros technology (2-3 μm).[105] Additionally, these particles were smaller than those produced using AIR technology (5-30 μm).[106] Based on Equation 1, our data suggest a mean shape factor equal to 0.14 (assuming a ρ_{ref} of 1 mg/μL and $\rho_{tap} = \rho_{particle}$). This value is much less

than 1, indicating that our particles are aspherical and highly irregular in morphology, thus making them good candidates for inhalation. This observation is further corroborated by SEM imaging (Figure 2).

Circular dichroism was used to determine changes in the secondary structure of insulin that might occur as a result of particle processing. The data suggest that there are no irreversible changes that occur as a result of processing, and that the thermal stability of the insulin processed into microparticles and nanoparticles is neither enhanced nor diminished (Figure 5). This suggests that the activity of the protein is retained throughout each step of the formulation.

The crystallinity of the insulin particles was first examined using 13C CP/MAS NMR (Figure 6). Due to their highly ordered nature, crystalline materials will have relatively narrow lines in a 13C CP/MAS spectrum, while disordered or amorphous materials have relatively broad lines. Insulin consists of 51 amino acids and therefore the spectrum will be quite complicated because every amino acid will have at least an amide and a carbon, each of which will have slightly different conformations and thus different chemical shifts. Because of this, even the 13C CP/MAS spectrum of a crystalline protein will appear to have broad lines even though it may actually be composed of many narrow lines with slightly different chemical shifts. Therefore, it would be expected that there would be very few differences between the 13C CP/MAS spectra of amorphous and crystalline

proteins, and any differences may be subtle. The sharply resolved peaks of the unprocessed insulin would suggest that it is crystalline while all of the other samples appear to be amorphous, which is corroborated by the dissolution testing. However, at this time nothing can be said about the purity of each form because there could be some crystalline insulin in the samples that appears to be amorphous.

Crystallinity was also determined by dissolution testing, as defined by the 2005 U.S. Pharmacopoeia National Formulary, with modifications. Buffered acetone TS was used to dissolve the amorphous insulin from each sample, the concentration of which was then determined and used to estimate the crystallinity of the particles. The unprocessed insulin was shown to be about 17 times more crystalline than the nanoparticles, and 4 times more crystalline than the microparticles (Figure 7). The dissolution rate of both the microparticles and the nanoparticles exhibited a burst effect over the first few minutes when compared to the unprocessed insulin (Figure 8). This burst may be due to the rapid dissolution of amorphous material deposited on the surface of the particles during processing or possibly during lyophilization. In the case of the nanoparticles, it is probable that the large total surface area of the particles also plays a significant role in increasing the dissolution rate. This may be beneficial in a pulmonary insulin formulation if the desired therapeutic effect is rapid control of spikes in glucose levels. This type of

formulation may be adjusted for sustained control of glucose over long periods of time, or for postprandial glucose control.

One concern with the technique outlined in the U.S. Pharmacopoeia is that it is based on the assumption that amorphous materials dissolve more rapidly than crystalline materials. This assumption might not always apply when using nanoparticles because of the enhanced dissolution velocity of particles at this scale, so interpretation of the results should be approached conservatively.[29,107] Additionally, it would be prudent to examine the shelf-life of the formulation using an accelerated stability study. Solid-state drug formulations are typically more thermodynamically stable in their crystalline forms than in their amorphous forms, which could cause the microparticle formulation to crystallize and possibly lose its burst-release properties over time. Future studies should also include examination of the formulation performance *in vivo* to determine the relative performance of this formulation over intravenous and subcutaneous insulin formulations, as well as other pulmonary insulin formulations.

2.4. Conclusions

Pure insulin microparticles produced through agglomeration of insulin nanoparticles may be a potential candidate for a pulmonary insulin formulation. The lack of penetration enhancers and other excipients in this formulation may reduce the occurrence of unforeseen side effects, thus

making it potentially safer than existing alternatives. Additionally, the processing steps necessary for this formulation are minimal and did not denature or degrade the peptide, which may be a concern with currently used techniques. A major benefit of this formulation method is the ability to produce pure insulin microparticles, which has not been demonstrated with techniques such as spray drying. The development of a dry powder insulin formulation for the treatment of diabetes may help to reduce, or altogether eliminate, patient dependence on painful injections, and thus may help to increase patient compliance.

3. References

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